Developmental Cell, Vol. 7, 897–907, December, 2004, Copyright ©2004 by Cell Press

The Apical Caspase *dronc* Governs Programmed and Unprogrammed Cell Death in *Drosophila*

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Summary

Among the seven caspases encoded in the fly genome, only *dronc* contains a caspase recruitment domain. To assess the function of this gene in development, we produced a null mutation in *dronc*. Animals lacking zygotic *dronc* are defective for programmed cell death (PCD) and arrest as early pupae. These mutants present a range of defects, including extensive hyperplasia of hematopoietic tissues, supernumerary neuronal cells, and head involution failure. *dronc* genetically interacts with the *Ced4*/*Apaf1* counterpart, *Dark*, and adult structures lacking *dronc* are disrupted for fine patterning. Furthermore, in diverse models of metabolic injury, *dronc*$^-$ cells are completely insensitive to induction of cell killing. These findings establish *dronc* as an essential regulator of cell number in development and illustrate broad requirements for this apical caspase in adaptive responses during stress-induced apoptosis.

Introduction

Caspases (cysteinyl aspartate-specific proteinases) are synthesized as dormant proenzymes. During apoptosis, these precursors are processed to their active form by complex networks of proteolytic activation (Danial and Korsmeyer, 2004; Salvesen and Duckett, 2002; Shi, 2002). The fly genome encodes seven members of the caspase family (Kumar and Doumanis, 2000). Based on prodomain structure, two of these, DREDD (Chen et al., 1998) and DRONC (Dorstyn et al., 1999), resemble apical or initiator caspases; the former contains death effector-like domains (DEDs) and the latter contains a caspase recruitment domain (CARD). Interactions between CARD containing caspases, adaptors, and inhibitor proteins characterize essential steps in apoptotic regulation (Hofmann and Bucher, 1997). A third caspase, STRICA, contains an unusual serine/threonine-rich prodomain, and the remaining four caspases (DCP1, DRICE, DECAY, and DAMM) resemble executioner caspases, with notably short prodomains. The precise cleavage relationships among these are often inferred and much remains to be learned regarding the function of each.

In any given cell, opposing regulatory forces determine the status of caspase activity (Salvesen and Abrams, 2004). For example, activation of apical caspases requires physical interactions with positive acting adaptor proteins that recruit unprocessed zymogens (Green, 1998; Salvesen, 2002). In *Drosophila*, this function is encoded by DARK (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999), a protein homologous to CED-4 in the worm and APAF-1 in mammals. DARK shares a C-terminal WD domain in common with APAF-1, and in this respect, both the fly and mammalian genes are distinct from their worm counterpart, which lacks this domain. In mammals and in insects, caspases are also subject to negative control by members of the IAP gene family (Salvesen and Abrams, 2004), which contain a characteristic motif referred to as the BIR domain (Crook et al., 1993). Of the *Drosophila* BIR-containing genes tested so far, DIAP1 appears to be a central regulator of apoptosis (Hay, 2000). Through direct interactions, DIAP1 inhibits DRICE (Kaiser et al., 1998; Wang et al., 1999) and DRONC (Meier et al., 2000b) and may also target itself and DRONC (Muro et al., 2002; Wilson et al., 2002) for ubiquitination-mediated degradation (Chai et al., 2003). In flies, all programmed cell death (PCD) in the embryo is specified through proteins (*Rpr*, *Grim*, *Hid*) that act, in part, by antagonizing DIAP1 (Abrams, 1999; Baehrecke, 2002; Meier et al., 2000a; Yin and Thummel, 2004).

Studies that examine the functional properties of *Drosophila* caspases have primarily relied on multigenic deficiencies, dominant-negative variants (Meier et al., 2000b), or injected dsRNAs (Quinn et al., 2000). Genetic studies have established requirements for *dredd* in innate immunity (Leulier et al., 2000) and a role for *dcp1* in stress-induced death in the ovary (Laundrie et al., 2003). As the only CARD domain-containing caspase encoded in the fly genome, *dronc* is thought to be crucial for PCD (Dorstyn et al., 1999; Meier et al., 2000b). Support for this inference includes detection of DRONC/DARK complexes (Dorstyn et al., 2002; Quinn et al., 2000), RNAi studies (Quinn et al., 2000), and dominant-negative transgenes that, in recent studies, have also implicated *dronc* in functions unrelated to PCD (Huh et al., 2004b). Mutations in *dronc* have not been isolated, and hence, the actual requirements for this apical caspase in development and in PCD are not known. To examine these issues, we conducted genetic analyses of this locus and established *dronc* as a recessive, lethal gene. Animals lacking a zygotic source of *dronc* arrested as early pupae, were defective for PCD in some tissues, and showed a genetic interaction with *dark*. These mutants exhibit a range of defects, including severe hyperplasia of blood cells, supernumerary neuronal cells, and head involution failure. In clonal analyses, we found that

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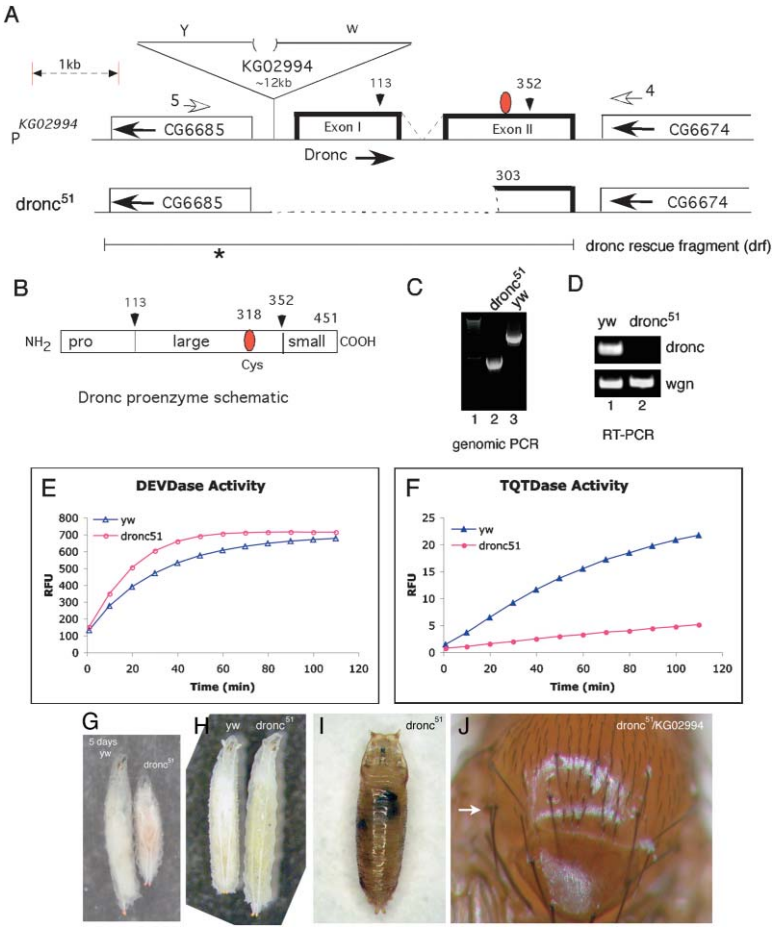


Figure 1. The *dronc*^{51} Allele Is a Single Gene Deletion

(A) Schematized genomic structure at the *dronc* locus. Included are the P insertion, *KG02994*; *dronc*^{51}, a partial deletion at *dronc*, and positions of relevant primers (open arrows). *KG02994* is a transposon that maps 5′-ward of *dronc* (top). *dronc*^{51} (bottom) excises the P element, producing a deletion that eliminates sequences from the P insertion site to codon 303 of the *dronc* ORF. The deletion (indicated by the dashed line) eliminates the P element and removes sequences downstream of this insertion, deleting most of the *dronc* locus. Note that this allele leaves sequences upstream of the original P insertion completely intact. A genomic fragment (indicated) containing the wild-type *dronc* locus and sequences spanning *CG6685*, but mutated for the *CG6685* ORF, was introduced into *dronc*^{51} animals. Asterisk denotes position of frameshift mutation in *CG6685*. This *dronc rescue fragment*, designated *drf*, is described in detail in the accompanying paper from Daish et al. (2004). *dronc*^{51} animals carrying this transgene are referred to as *dronc*$^{51:drf}$.

(B) Domain structure of the DRONC protein, indicating the active site cysteine (red oval).

(C) Genomic PCR with primers 4 and 5 (open arrows, [A]), detects a deletion in *dronc*^{51}/*KG02994* animals. The mutant band was fully sequenced, validating the map as in (A).

(D) RT-PCR reactions with a primer 3′-ward of deleted *dronc* seqences were performed to confirm that *dronc* RNAs are not expressed in *dronc*^{51} homozygous larvae. Detection of *wgn* (Kauppila et al., 2003) is used here as a control.

(E and F) DEVDase and TQTDase activities in L3 larval extracts. Wild-type (*yw*) and homozygous *dronc*^{51} extracts were prepared, and DEVDase and TQTDase activities were measured. Multiple experiments were conducted. Results shown are the average readings of triplicate samples from one representative experiment. RFU, relative fluorescent units.

(G) Size comparisons of wild-type (*yw*) and *dronc*^{51} larvae at day 5.

(H) Size comparisons of *dronc*^{51} and wild-type wandering L3 larvae. At the late wandering L3 larval stage, ~90% of *dronc*^{51} homozygotes are noticeably larger than their wild-type counterparts.

(I) *dronc*^{51} early pupa. Note that *dronc*^{51} pupae have occasional melanotic tissues as shown. Also note that *dronc*^{51} pupae never proceed beyond early pupal stage.

(J) Extra scutellar bristle phenotype (arrow) seen on *dronc*^{51}/*KG02994* adults (see Table 1).

adult tissues were also affected, since fine patterning of the eye was disrupted and a progressive, age-dependent degeneration of wing tissue occurred. Furthermore, in diverse models of metabolic injury, *dronc*$^{-}$ cells were completely insensitive to induction of cell killing. These studies establish *dronc* as an essential regulator of cell number in development and reveal broad requirements for this apical caspase in adaptive responses during stress-induced apoptosis.

Results

Alleles at the *dronc* Locus: *dronc*^{51} Is a Null and P^{KG02994} Is a Hypomorph

To characterize the *dronc* locus, we screened for null mutations in this gene. Our strategy involved mobilization of a P transposon insertion, P^{KG02994}, mapping 113 bp upstream of the *dronc* transcription start site (Figure 1). Line 51 (*dronc*^{51}) was identified by genomic PCR screens (Figure 1C) and validated by sequence analyses. The mutation is an imprecise excision of P^{KG02994} that deletes sequences downstream of the transposon through most of the *dronc* locus, leaving all sequences upstream of the P^{KG02994} insertion site intact and unaffected (Figure 1A). To further confirm that *dronc*^{51} is a null allele, we performed RT-PCR using primers spanning the remaining region of *dronc* mRNA and, as illustrated in Figure 1D, no *dronc* transcript was detected. We also measured caspase activity in larval extracts using a preferred substrate for DRONC, TQTD, and another substrate, DEVD, which detects caspase-3-like activity (Figures 1E and 1F). As expected, TQTDase activity was dramatically decreased in *dronc*^{51} extracts compared to wild-type controls (*yw*). In contrast, DEVDase activity was not affected in *dronc*^{51} mutants.

Homozygous *dronc*^{51} animals are not viable. Therefore, we closely followed development of these individuals from embryonic to adult stages to determine the lethal phase. Despite a head involution defect seen in

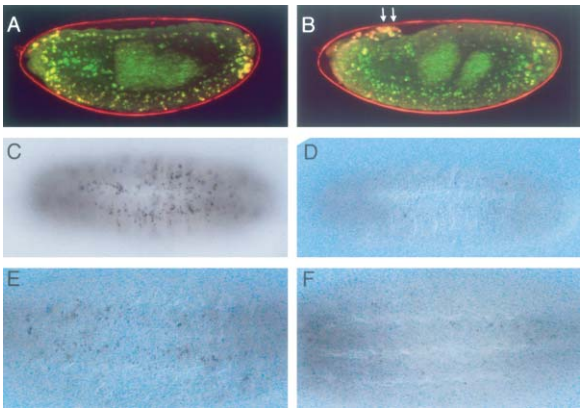


Figure 2. *dronc* Embryos Are Defective for Programmed Cell Death and Head Involution

Embryos were stained with acridine orange (A and B) and TUNEL (C–F) to visualize PCD. Note that $dronc^{51}$ embryos shown here were all progeny of $dronc^{51}/KG02994$ female flies.

(A and B) Comparably staged wild-type *yw* (A) and $dronc^{51}$ (B) embryos. The arrows in (B) indicate ectopic tissue, persisting from the dorsal ridge, which is characteristic for the head involution defect (see also Figure 3B).

(C and D) Ventral views of late stage 12 *yw* (C) and $dronc^{51}$ (D) embryos, showing the incidence of apoptosis detected by TUNEL in the epidermis.

(E and F) TUNEL labeling in the ventral nerve cord of stage 15/16 *yw* (E) and $dronc^{51}$ (F) embryos.

late embryogenesis (Figures 2 and 3), more than 98% of $dronc^{51}$ embryos hatched within 36 hr after egg laying. Similar results were observed when $dronc^{51}$ was placed in *trans* to a chromosomal deficiency that uncovers *dronc*, Df(3L)Ac1. Postembryonic growth of $dronc^{51}$ animals was delayed through the third instar phase and the latest lethal phase occurred in the early pupal stage (Figure 1I; see also Daish et al., 2004 [this issue of *Developmental Cell*]). When compared to wild-type larvae of the same chronologic age, $dronc^{51}$ L3 larvae were visibly smaller (Figure 1G), but older $dronc^{51}$ larvae can attain sizes larger than their wild-type counterparts (Figure 1H). Among late wandering third instar larvae, ~90% of $dronc^{51}$ homozygotes are noticeably larger than their wild-type counterparts. A computed gene, *CG6685*, mapping to the left of the $P^{KG02994}$ insertion was also examined in the $dronc^{51}$ allele. Using genomic and RT-PCR analyses, we confirmed that *CG6685* coding sequences are unaffected and found that *CG6685* is expressed, albeit at reduced levels, in $dronc^{51}$ larvae. To exclude possible hypomorphic *CG6685* activity in the context of subsequent studies, we conducted rescue experiments and found that a transgenic fragment wild-type for only *dronc* complemented the $dronc^{51}$ mutation in several respects (Figure 1A and Complementation section of Experimental Procedures; $dronc^{51}$ homozygotes with this *dronc* rescue fragment, designated *drf*, are referred to as $dronc^{51:drf}$ animals). First, the *drf* transgene complemented $dronc^{51}$ lethality, producing viable $dronc^{51}$ adults that were morphologically normal. As often found in transgenic complementation experiments (e.g., Ashburner, 1989; Letsou et al., 1993; Mohler et al., 1989), rescue of $dronc^{51}$ lethality was not complete, since expected Mendelian ratios were not observed and only occasional $dronc^{51:drf}$ flies were fully rescued through eclosion. In a second manifestation of rescue, we found that up to 50% of $dronc^{51:drf}$ animals became pharate adults, representing developmental progression ~3 days beyond the point at which $dronc^{51}$ homozygotes arrest. Complementation also occurred at the transition between second and third instar stages. For example, at 5 days of age, when 40% of $dronc^{51}$ homozygotes were still in the second instar larval phase, more than 95% of the $dronc^{51:drf}$ animals had become third instars, with demographics indistinguishable from wild-type populations. Likewise, at 6 days of age, when 100% of the $dronc^{51:drf}$ animals had become third instar larva, at least 37% of $dronc^{51}$ homozygotes were still in the second instar stage. Finally, it is worth noting here that complementation was also established at the biochemical level (Figure 6G) and, consistent with partial rescue, the drf transgene restored caspase activity to ~50% of wild-type levels (Figure 6G).

Additional genetic analyses (Table 1) uncovered maternal effect phenotypes at *dronc* and established that $P^{KG02994}$ is a hypomorphic allele at this locus. For example, the penetrance of a characteristic "extra macrochaete" phenotype seen in $P^{KG02994}$ homozygotes (Figure 1J) is exaggerated in $dronc^{51}/P^{KG02994}$ animals, and similarly, $dronc^{51}$ homozygous embryos produced by $dronc^{51}/P^{KG02994}$ females did not hatch. Furthermore, these progeny showed more severe PCD defects and elevated penetrance of head involution failure when compared to $dronc^{51}$ homozygotes derived from $dronc^{51}/+$ mothers (Figures 2 and 3). Notably, these phenotypes were not observed when two precise excision revertant lines, $dronc^{rev78}$ and $dronc^{rev79}$, were tested in similar crosses.

Zygotic *dronc* Is Required for PCD

To determine the role of *dronc* in programmed cell death, we examined apoptosis in $dronc^{51}$ homozygous embryos using acridine orange staining (Abrams et al., 1993) or TUNEL (White et al., 1994). If collected from parents heterozygous for this allele, levels of apoptosis in $dronc^{51}$ homozygotes were indistinguishable from wild-type but, like other cell death-defective mutants (Grether et al., 1995; White et al., 1994), a failure in head involution was clearly evident (not shown). We next checked homozygous $dronc^{51}$ embryos laid by $dronc^{51}/P^{KG02994}$ parents, since these were predicted to contain less maternally loaded product. Maternally compromised $dronc^{51}$ embryos exhibit severe head involution defects (Figures 2B and 3B), but acridine staining did not expose significant differences between wild-type and homozygous mutants (Figures 2A and 2B). However, a more highly resolved analysis using TUNEL to label apoptotic cells showed a marked reduction of PCD in $dronc^{51}$ embryos. This phenotype was particularly evident in the ventral epidermis of early embryos and in the condensing ventral nerve cord of older embryos, where considerably reduced levels of apoptosis are seen (Figure 2, compare C to D and E to F).

Additional Cells Persist in $dronc^{51}$ Homozygotes

Cells that fail to die often adopt differentiated fates, and therefore, PCD defects typically produce supernumerary cells (Ellis et al., 1991; Rodriguez et al., 1999; White et

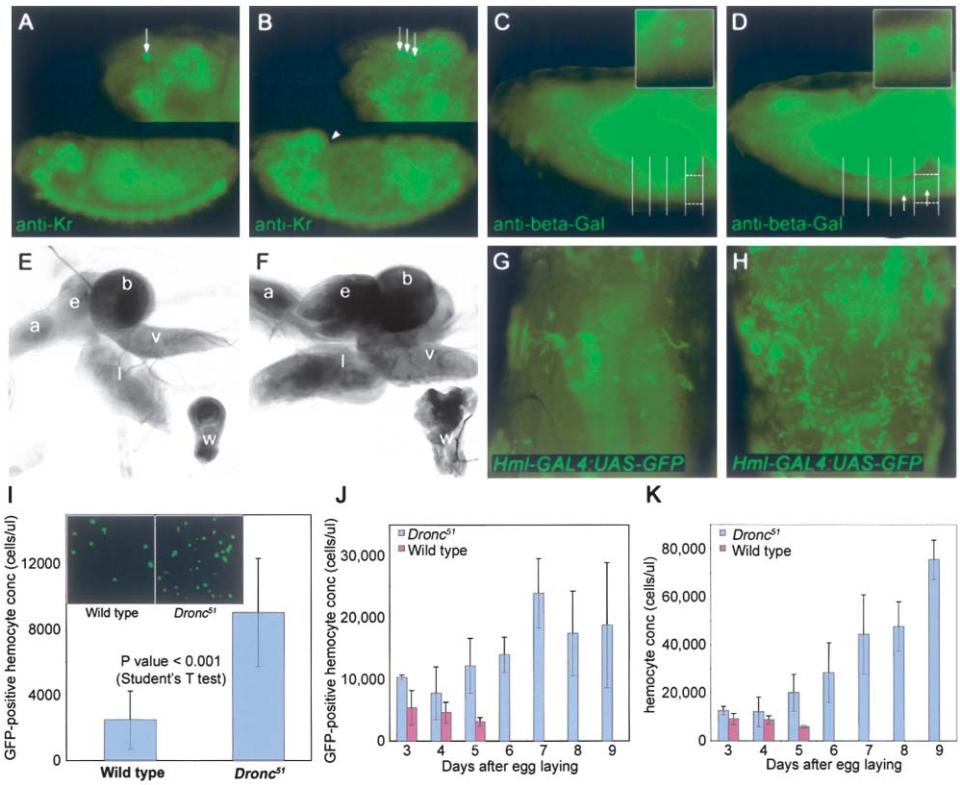


Figure 3. *dronc*^{51} Animals Have Additional Cells

(A and B) Stage 15 embryos labeled with α-kruppel antibody. Insets show enlarged dorsoanterior region of the same embryo.

(A) Wild-type (*yw*) embryo with *Kruppel* immunoreactivity indicated in the optic organ (thin arrow).

(B) *dronc*^{51} embryo with extra cells associated with the optic organ (arrows) and dorsal ridge abnormality associated with head involution defect (arrowhead, also see Figure 2B).

(C and D) Late-stage embryos expressing the *p[1.0Slit-LacZ]* marker stained with α-β-Gal antibody to label *Slit*-positive cells. Wild-type embryo (C) and comparably staged *p[1.0Slit-LacZ]; dronc*^{51} embryo (D) are shown with segmental boundaries in the nerve cord (white lines). Insets show a single segment from the nerve cord of the same animal (boxed). Note that 2–3 *Slit-LacZ*-positive cells are seen in each segment of wild-type animals, whereas some segments of *p[1.0Slit-LacZ]; dronc*^{51} embryos show 4–5 *Slit-LacZ*-expressing cells (arrows in D). Inset in (D) shows a single segment with 4 *Slit-LacZ*-positive cells.

(E and F) *yw* (E) and *dronc*^{51} (F) wandering third instar larvae dissected to show the major central nervous system organs and imaginal discs. Abbreviations: a, antenna disc; e, eye disc; b, brain; l, thoracic leg disc; v, ventral nerve ganglion; w, wing disc (inset).

(G–K) Hyperplasia of blood cells in *dronc* mutant animals was detected using *Hml-GAL4:UAS-GFP*, a marker chromosome for hemocytes.

(G and H) Whole-mount wild-type (G) and *dronc*^{51} (H) wandering third instar larva showing GFP-labeled hemocytes through the transparent body wall.

(I) GFP-expressing hemocyte concentration in wild-type and *dronc*^{51} wandering third instar larvae (determined from hemolymph aspirates as described in Experimental Procedures). Insets are low-magnification views of the GFP-expressing hemocytes from hemolymph slide spreads.

(J and K) GFP-positive (J) and total (K) hemocyte concentration as a function of age.

Error bars denote standard deviation. Note in (A)–(D), (G), and (H), fluorescence associated with gut is nonspecific.

al., 1994). We used several markers to examine *dronc*^{51} mutants for persisting "extra cells." In studies with α-Kruppel antibody (Kosman et al., 1998), we found that *dronc*^{51} embryos have additional cells associated with the optic organ (Figures 3A and 3B). Together with head involution failures (Figures 2B and 3B), these phenotypes closely resemble mutations in another PCD locus, *hid* (Grether et al., 1995). Extra cells expressing a Slit-LacZ reporter were also observed in the midline of the embryonic central nervous system (CNS). Normally, 2–3 slit-expressing midline glial cells are detected within each segment (Rodriguez et al., 2002a; Zhou et al., 1995) but, in *dronc*^{51} mutants, we observed segments containing four or more *slit*-positive cells (Figures 3C and 3D).

In wandering third instar larva, *dronc*^{51} animals often exhibit abnormalities of internal structures. For example, the brain lobes of these mutants are typically larger than wild-type counterparts. Likewise, the imaginal discs are often enlarged and the wing discs are frequently deformed (Figures 3E and 3F). The severity of these phenotypes strongly correlated with larval size such that, in a given population of *dronc*^{51} animals, these phenotypes were noticeably more severe among the larger sized larva. Despite these irregularities, the progression of the morphogenetic furrow through the eye disc appears unaffected in *dronc*^{51} animals (Figures 4E and 4F), suggesting that proper adult differentiation programs are initiated. We also examined hemocytes in third instar larva and discovered conspicuous hyperplasia of blood cells in *dronc*^{51}. To facilitate quantitative assessments of this phenotype, hemocytes were labeled with green fluorescent protein (GFP) using the *Hml-GAL4:UAS-GFP*

Table 1. *dronc*^{51} Is a Null, and *KG02994* Is a Hypomorphic Allele of dronc

Genotype	Viable	Extra Bristles (N)
dronc^{51}/*dronc*^{51}	no[a]	
dronc^{51}/+	yes	0% (85)
dronc^{51}/*KG02994*	yes[b]	12% (352)
KG02994/+	yes	0.5% (206)
KG02994/*KG02994*	yes	4% (101)
dronc^{51}/*Df(3L)Ac1*	no	
KG02994/*Df(3L)Ac1*	yes	ND
dronc^{rev78}/*Df(3L)Ac1*	yes[c]	ND

[a] *dronc*^{51} homozygous animals can survive to pupal stage (see Figure 1) when genotypes of both parents are *dronc*^{51}/+ or *dronc*^{51}/*TM3*. However if the maternal genotype is *dronc*^{51}/*KG02994*, then *dronc*^{51} homozygous progeny die as late embryos.
[b] Note *dronc*^{51}/*KG02994* animals exhibit increased penetrance of extra bristle phenotype compared to *KG02994* homozygotes. In addition, *dronc*^{51}/*KG02994* progeny are not viable when their mother's genotype is *dronc*^{51}/*KG02994*.
[c] *dronc*^{rev78} is a precise excision revertant of the P element *KG02994*.

marker chromosome. When visualized with this marker in vivo, *dronc*^{51} animals show radical hyperplasia of blood cells at the wandering third instar larval stage (Figures 3G and 3H). As described in the Experimental Procedures section, we also conducted ex vivo counts of GFP$^+$ hemocytes, where more than a 3-fold elevation of density was found (Figure 3I). Since hemolymph aspirates sample only circulating but not sessile blood cells, we suspect that differences represented here (Figures 3I–3K) actually produce an underestimate of the true extent of hyperplasia. Similar counts of total or GFP-expressing blood cells in aged third instar larvae (Figures 3J and 3K) indicated that significant elevations can be detected as early as 5 days after egg laying (AEL). In animals homozygous for the *Hml-GAL4:UAS-GFP* marker chromosome, about half of all hemocytes are negative for GFP, and it is possible that GFP$^+$ hemocytes were amplified at the expense of GFP$^-$ hemocytes. Two findings, however, exclude this formal possibility. First, comparable estimates for the degree of hyperplasia in *dronc*^{51} animals were obtained in counts of total (Figure 3K) or GFP$^+$ hemocytes (Figure 3J). Second, flow cytometry studies (Figure 6) established that the ratio of GFP$^-$ to GFP$^+$ hemocytes is unaffected in *dronc*^{51} (not shown). Therefore, elevated blood cell counts in *dronc*^{51} larvae derive from absolute increases in cell number, rather than redirected fates among hemocyte subpopulations.

dronc Is Required for Fine Patterning of the Adult Eye

To examine requirements for *dronc* action in the formation of adult tissues, we used a FLP-FRT-based recombination strategy (Stowers and Schwarz, 1999) to produce eyes made exclusively from *dronc*^{51} homozygous cells. Our strategy was authenticated, in part, by the appearance of rare individuals where only one of two eyes underwent FLP-induced recombination (Figure 4D). At the gross level, *dronc*$^-$ eyes had normal size and overall structure, but at a finer scale, these showed a "rough" phenotype (Figure 4C) when compared to wild-type (Figure 4A) and FLP/FRT (Figure 4B) controls. This defect indicates that loss of *dronc* somehow disrupts normal developmental patterning. We can exclude extra photoreceptor cells and/or abnormal ommatidial size as the basis for this phenotype, since these features appeared normal in plastic sections of *dronc*$^-$ eyes (not shown). Therefore, it is likely that cell types not examined here are affected. To elucidate the earliest requirement for *dronc* in the developing eye, we stained eye discs from *dronc*^{51} wandering third instars with phalloidin and α-Boss antibody (Figures 4E and 4F). In the absence of *dronc*, the morphogenetic furrow formed and progressed normally, but many irregularly shaped preclusters occurred and Boss-expressing cells were often unevenly spaced. Therefore, *dronc* functions at, or prior to, events that serve to organize preclusters in the developing eye.

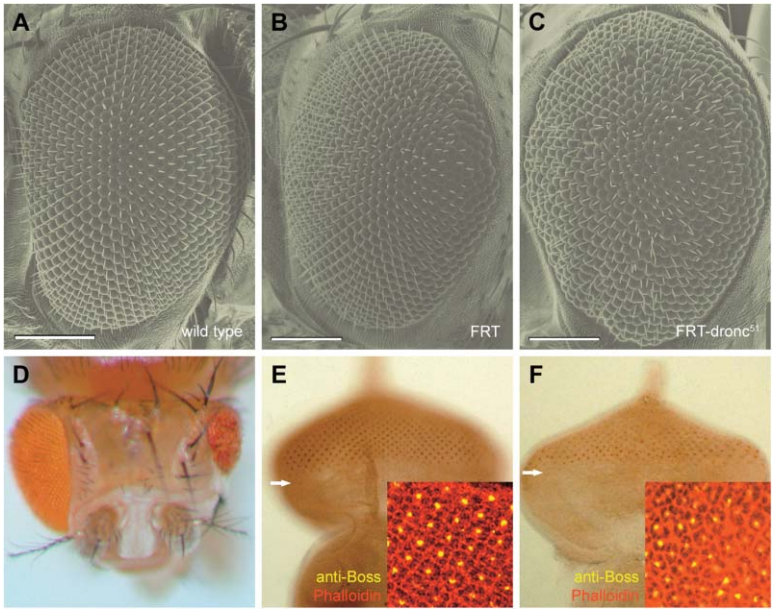


Figure 4. *dronc* Is Required for Fine Patterning of the Retina

(A–C) Whole eyes lacking *dronc* were produced using *FLP/FRT* recombination together with *eyeless* selection (Stowers and Schwarz, 1999). Scanning electron micrographs of eyes shown are (A) parental wild-type control, (B) *FLP/FRT* control, and (C) *FLP/FRT-dronc*^{51}. Note increased "roughness" in (C) compared to (B). Also note that genotype of animals (see Experimental Procedures) in (B) and (C) are identical except for the *dronc*^{51} chromosome arm.

(D) Example of rare animal where only one eye (larger) underwent mitotic recombination, but the other eye did not.

(E and F) Eye discs from *yw* (E) and *dronc*^{51} (F) wandering third instar larvae probed with antibody against *Bride of Sevenless* (*Boss*). Insets show close-up of cells double-labeled with α-Boss antibody and phalloidin (which stains the actin cytoskeleton). Arrow shows approximate position of morphogenetic furrow progressing from posterior (top) to anterior (bottom).

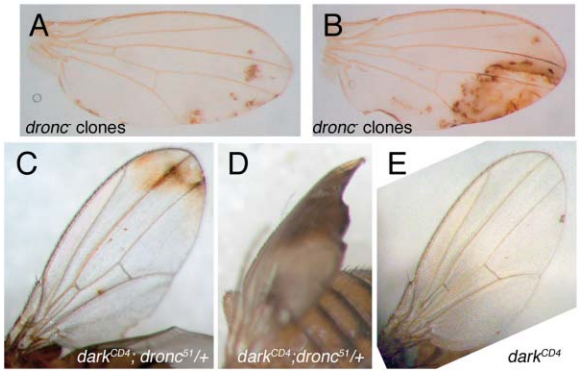


Figure 5. Requirement for *dronc* and Its Interaction with *Dark* in the Adult Wing

(A and B) Wings mosaic for $dronc^{51}$. Genotype is *vg:flp/+; $dronc^{51}$, FRT79/FRT79* (see Experimental Procedures). Note that wings are normal at eclosion and blemishes appear after 4 days. Pictured here are wings at 7 days.

(C and D) Animals heterozygous for $dronc^{51}$ exhibit similar adult onset blemishes only if placed in a genetic background homozygous for a hypomorphic allele of *Dark* ($Dark^{CD4}$). Note that wings of flies heterozygous for $dronc^{51}$ never exhibit this phenotype.

(E) Wing of 7-day-old $dark^{CD4}$ homozygote. Note blemishes also occur but these are markedly less severe.

The DRONC/DARK Axis Is Required for Proper Maintenance of Adult Wing Tissue

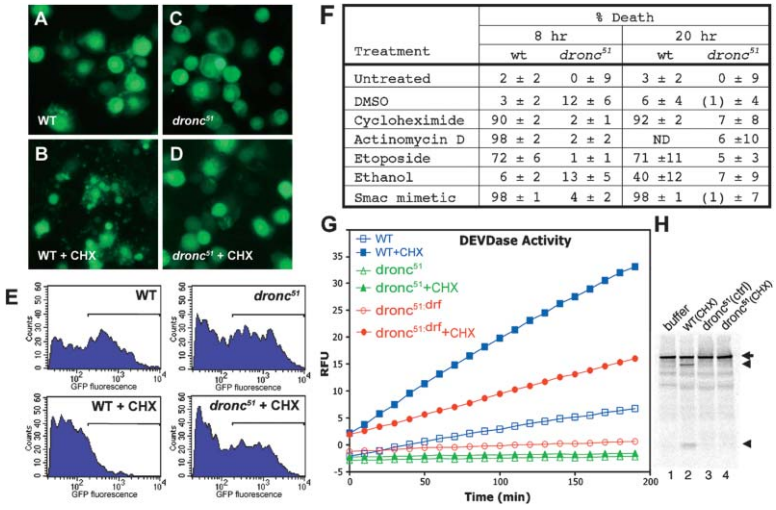
To further examine *dronc* function in the formation of adult tissues, we produced $dronc^{51}$ clones in the wing. At eclosion, adult wings mosaic for $dronc^{51}$ were morphologically normal and indistinguishable from wild-type. Therefore, *dronc* function is not required for normal formation of wing tissue. However, within 4 days, melanized blemishes appeared at random positions throughout the wing of all mosaic animals (Figures 5A and 5B). Although wing abnormalities are hardly uncommon, to our knowledge, all previously described wing phenotypes are congenital (existing at eclosion). Therefore, the progressive and age-dependent nature of these blemishes is quite unusual. Additional significance can be assigned to this phenotype because similar, yet less severe, wing blemishes are also observed in adults homozygous for $dark^{CD4}$, which is a hypomorphic allele of the *Apaf-1/Ced4* counterpart (Figure 5E; Rodriguez et al., 1999). Moreover, when the dosage of *dronc* was reduced by half in $dark^{CD4}$ adults, the melanized wing blemishes became far more severe (Figures 5C–5E). This genetic interaction is distinctive and specific since numerous other mutants showed no such effects in combination with $dark^{CD4}$ and, importantly, wing defects were never observed in $dronc^{51}$ heterozygous flies. These findings uncover unappreciated roles for these genes in the maintenance of adult tissue and, in this respect, *dronc* qualifies as a classic enhancer of *dark*.

dronc Is Required for Stress-Induced Apoptosis

To examine the function of *dronc* in unprogrammed cell death, we measured responses in ex vivo cultures of larval hemocytes treated with proapoptotic agents. In stark contrast to wild-type hemocytes, which were acutely reactive to these stressors, $dronc^{51}$ hemocytes were remarkably insensitive. For example, cycloheximide (CHX) triggers widespread apoptosis in hemocytes from wild-type animals within 8 hr (Figures 6A, 6B, and 6E), yet no apoptosis is detected in hemocytes from $dronc^{51}$ animals (Figures 6C–6E), even when 10-fold higher concentrations of CHX were applied (not shown). This effect was not simply a delay in the kinetics of apoptosis since $dronc^{51}$ cells were equally unresponsive at 20 hr and, even after 48 hr in cycloheximide, $dronc^{51}$ cells were evidently alive and continued to exhibit changes in shape. Resistance to proapoptotic agents was not confined to protein synthesis inhibitors. For example, marked insensitivity was also documented in $dronc^{51}$ hemocytes after exposure to Actinomycin D (transcription inhibitor), etoposide (Topoisomerase II inhibitor), ethanol, and a small molecule Smac mimetic (Li et al., 2004) (Figure 6F). In the course of these studies, we also noticed that constitutive apoptosis in these primary cultures was influenced by the absence of *dronc*. For instance, at any given time, 2%–3% of hemocytes from wild-type animals were typically apoptotic. In contrast, apoptotic hemocytes from $dronc^{51}$ animals were exceptionally rare in these ex vivo preparations (note that statistical indicators of percent death in $dronc^{51}$ samples arise predominantly, if not exclusively, from variance in plating efficiencies). Together, these results establish a pivotal role for *dronc* as a mediator of stress-induced apoptosis. To substantiate these findings, we extended these studies using two enzymatic assays to detect effector caspase activity (Figures 6G and 6H). When stimulated with CHX, hemocytes from wild-type or $dronc^{51:drf}$ rescued animals show pronounced induction of DEVDase activity. In stark contrast, hemocytes derived from $dronc^{51}$ animals were completely devoid of DEVDase (Figure 6G), and in a similar assay, they were also negative when measured for PARP cleavage activity (Figure 6H). While these data directly establish a fundamental role for Dronc in the context of stress-induced effector caspase activation, they also highlight two additional findings worth noting here. First, using this measure of genetic activity, the drf transgene restores some, but not all, of wild-type *dronc* function. Second, in contrast to lysates from hemocytes, whole larval lysates were not defective for DEVDase activity (Figure 1E). Together, these findings argue that distinct, tissue-specific pathways of effector caspase activation exist in *Drosophila*, some of which are Dronc dependent and some of which are Dronc independent.

Discussion

Of the three apical caspases found in the fly genome, only *dronc* contains a CARD domain. Analyses of *dronc* alleles presented here, together with independent alleles described in the accompanying article from Daish et al. (2004), establish essential functions for this caspase in PCD. Zygotically expressed *dronc* is absolutely required to progress beyond the early pupal stage. In addition, we found conspicuous parallels to phenotypes associated with other cell death regulators in the fly. For example, head involution failures in $dronc^{51}$ embryos resemble phenotypes associated with *hid*, a prominent apoptosis activator mapping to the *Reaper* region (Grether et al., 1995). Likewise, the extra scutellar bristles seen with viable combinations of *dronc* alleles (Figure 1J) are also seen in animals mutated for *dark* (Rodriguez et al., 1999)



F

Treatment	% Death 8 hr wt	8 hr *dronc*^{51}	20 hr wt	20 hr *dronc*^{51}
Untreated	2 ± 2	0 ± 9	3 ± 2	0 ± 9
DMSO	3 ± 2	12 ± 6	6 ± 4	(1) ± 4
Cycloheximide	90 ± 2	2 ± 1	92 ± 2	7 ± 8
Actinomycin D	98 ± 2	2 ± 2	ND	6 ±10
Etoposide	72 ± 6	1 ± 1	71 ±11	5 ± 3
Ethanol	6 ± 2	13 ± 5	40 ±12	7 ± 9
Smac mimetic	98 ± 1	4 ± 2	98 ± 1	(1) ± 7

Figure 6. *dronc* Is Required for Stress-Induced Apoptosis and Effector Caspase Activity

(A–D) Hemocyte aspirates expressing *Hml-GAL4:UAS-GFP* from wild-type and *dronc*^{51} larvae were challenged with various chemical stressors ex vivo. Cycloheximide (CHX)-induced apoptosis is readily seen in wild-type cells (compare A and B), but hemocytes from *dronc*^{51} animals are insensitive to this challenge (compare C and D).

(E) In different trials of the same experiment, flow cytometric analysis detects CHX-induced apoptosis as loss of GFP-expressing cells. Brackets denote viable cells. Note loss of this population in wild-type but not *dronc*^{51}.

(F) Quantification of apoptosis at 8 hr and 20 hr induced by the various agents indicated. Since measures of percent death were normalized to untreated wells and apoptosis of *dronc*^{51} hemocytes is extremely rare, note that cell death counts in *dronc*^{51} aspirates arise predominantly, if not exclusively, from variance in plating efficiencies. Percentages shown with standard deviations, and parentheses denote a negative number.

(G) DEVDase activity was measured in hemocyte lysates derived from larvae of genotypes indicated, treated with or without CHX. Note induction of activity in hemocytes from wild-type and the *dronc* rescue strain (drf), but no detectable activity occurs in lysates from *dronc*^{51} hemocytes under either condition. Also note that hemocytes derived from the *dronc* rescue strain (drf) exhibit ~50% wild-type activity. Data shown are representative of multiple trials. RFU, relative fluorescence units.

(H) In vitro translated [^{35}S]PARP was used as a substrate to detect caspase-3-like activities in CHX-treated hemocytes as labeled. The arrow indicates full-length PARP and arrowheads indicate cleavage products. Note PARP cleavage activity occurs in lysates from wild-type (wt) but not *dronc*^{51} hemocytes. ctrl, control.

or the apical caspase *dredd* (Rodriguez, 2002). These common phenotypes indicate shared apoptotic functions, highlighting the importance of caspase-dependent PCD as an arbiter of morphogenesis in the embryo and as a determinant of the number and position of mechanosensory organs in adults.

dronc mutations also share supernumerary cells in common with mutations at *dark* (Rodriguez et al., 1999), *hid*, and *grim* (Zhou et al., 1997). Moreover, it is well established that expression of proapoptotic genes in the *Reaper* region precedes PCD among cells labeled with the neuronal markers used here (*Kruppel* and *slit*), and therefore, at least some of the cells that fail to die in *dronc*^{51} animals require the induced action of these same PCD genes (Christich et al., 2002; Grether et al., 1995; White et al., 1994; Wing et al., 1998; Zhou et al., 1995). Consistent with this deduction, heterozygosity for *dronc* suppresses eye ablation phenotypes caused by *rpr*, *grim*, and *hid* (Meier et al., 2000b; Quinn et al., 2000; also see accompanying paper by Daish et al., 2004), and *dronc*$^{-}$ cells were completely insensitive to killing by a Smac mimetic compound (Figure 6F). Together, these observations favor models that place *dronc* downstream of IAP antagonists. If true, this model might not generalize to all dying cells since *dronc*^{51} embryos are only partially cell death defective, whereas all PCD in the developing embryo requires the combined action of IAP antagonists (*rpr, grim*, and *hid*). Therefore, during PCD, perhaps IAP antagonists have the capacity to engage activated forms of other fly caspases without a requirement for *dronc*. Consistent with this scenario, caspase-3-like activity was absent from *dronc*^{51} hemocytes (Figures 6G and 6H) but was not compromised in lysates from whole *dronc*^{51} larvae, where many tissues are simultaneously assayed (Figures 1E and 1F). These data argue against models proposing Dronc as the sole source of effector caspase activation in flies. Instead, our findings implicate tissue-specific pathways of effector caspase activation, only some of which qualify as strictly Dronc dependent. An alternative premise suggests that maternally supplied *dronc* provides a source of activity sufficient for much of prepupal development, and if so, perhaps *dronc* is needed for all PCD, analogous to *Ced-3* function in the worm (Horvitz, 1999). To directly examine this issue, and thereby distinguish these scenarios, we sought to examine animals lacking both maternal and zygotic *dronc*. However, we were unable to recover *Ovo*^{D}-derived germline clones, consistent with suggestions that the action of *dronc* is necessary for germline development (Geisbrecht and Montell, 2004). While maternal effects are clearly demonstrated from our genetic analyses, we nevertheless favor models for *dronc*-independent PCD pathways because apoptosis was evident in late larval stages when maternal supplies of *dronc* are probably negligible, diminished from effects of turnover and dilution. For example, wandering third instars homozygous for *dronc*^{51} were positive for TUNEL in the eye discs (not shown) and retained caspase-3-like activity (Figures 1E and 1F). Likewise, in the accompanying paper, Daish et al. (2004) find that, without *dronc*, PCD in the midgut occurs normally but is defective in the salivary gland and reduced in other tissues. Also, if *dronc*-independent pathways of effector caspase activation do occur, *dredd* is an unlikely contributor, since we found no evidence of genetic interactions between *dronc* and *dredd* (P.C. and J.M.A., unpublished observations).

From our studies thus far, hyperplasia of hematopoietic cells is among the more striking and unanticipated *dronc*^{51} phenotypes. Our results demonstrate that *dronc* is a fundamental regulator of blood cell number, but they do not fully establish the sources of dysregulation.

Since *dronc*$^{-}$ hemocytes are inherently resistant to apoptosis (Figure 6), it seems likely that autonomous failures in PCD contribute to this phenotype. Though we can exclude redirected cell fates as a source of extra cells, it is also possible that the absence of *dronc* encourages proliferation, directly or indirectly. Future studies could pinpoint cells within the hemocyte lineage where dysregulation first occurs. The magnitude of hyperplasia, together with demographic considerations, suggests that deregulated stem cells or progenitor cells, which have the ability to "amplify" the consequences of PCD failure, might best explain the *dronc*$^{-}$ phenotype, since preservation of cell types that lack proliferative potential would have only modest effects on total population numbers. At the same time, our results also establish that *dronc* profoundly governs apoptotic responses among mature blood cells as well. In fact, this apical caspase dictated sensitivities to a surprisingly broad range of insults, from widespread changes in translation (Cycloheximide) or transcription rates (Actinomycin D) to the likely focal impact of a small molecule Smac mimetic (Li et al., 2004). In addition, *dronc*$^{-}$ cells were completely insensitive in a model of genotoxic stress caused by Topoisomerase inhibition (etoposide). Therefore, like *caspase-9* in mammals (Zheng et al., 1999), distinct stress-responsive signaling networks must ultimately converge upon this apical caspase. It is especially noteworthy here that *dronc*$^{-}$ cells were also resistant when challenged by 1% ethanol (Figure 6). Since mechanisms of cell death in response to alcohols are largely unknown, continued studies in this model could highlight unique pathways connecting alcohol exposure and caspase activity.

Our analyses of mosaic tissues also exposed unanticipated roles for this caspase in the preservation and/or fine-scale patterning of adult tissues. Wings chimeric for *dronc* were grossly normal at eclosion and, therefore, *dronc* is not essential to form this tissue. However, within several days, *dronc*$^{-}$ cells prompted the appearance of melanized blemishes and these defects were markedly exacerbated by reduced *dark* gene action. This interaction argues that *dark* and *dronc* coordinately impact common cellular events, providing genetic confirmation of existing models for biochemical activities of these proteins (Danial and Korsmeyer, 2004). It is also worth noting that, to our knowledge, the progressive nature of the adult wing phenotype shown in Figure 5 is unusual and, consequently, the pathology responsible for this age-dependent defect is intriguing. One possible explanation may involve epidermal cells of the wing, which are effectively removed by PCD hours after eclosion (Kimura et al., 2004). Perhaps without proper *dronc* activity, failures in this process lead to patches of hypoxic cells that become melanized over a period of several days.

Retinal tissues produced exclusively from *dronc*$^{-}$ cells offer different but equally informative lessons. Since adult eyes lacking this caspase were grossly normal, we can exclude a requirement for *dronc* as an arbiter of overall size and structure in the formation of these organs. Given its insensitivity to the general inhibitor p35 (Hawkins et al., 2000; Meier et al., 2000b) and recently proposed nonapoptotic roles for this caspase in spermatid individualization (Huh et al., 2004b) and compensatory proliferation (Huh et al., 2004a), we emphasize that neither of these outcomes were obvious or predictable. Equally noteworthy, however, was a characteristic rough phenotype in whole eyes formed without *dronc*, which implicates important roles for this caspase in pattern formation at a finer scale. Once the precise origin of this defect is resolved, it will be interesting to determine whether the phenotype traces to cleavage of apoptotic or nonapoptotic targets.

Genetic studies described here, and complementary findings described in the accompanying paper from Daish et al. (2004), firmly establish zygotically expressed *dronc* as an essential effector of some, but not all, PCD pathways in the fly. In neuronal, hematopoietic, and perhaps other tissues, *dronc* also qualifies as a vital arbiter of cell number, and therefore, in principle, continued studies on these mutants could elucidate unknown signals that link tissue growth and cell number to caspase activity. Since *dronc* is also an essential determinant for many (if not all) stress-induced apoptosis pathways, similar strategies could illuminate unknown links between adaptive responses to stress and apoptotic physiology.

Experimental Procedures

Mutagenesis and Screen

KG02994 denotes *P[y+w+=SUPor-P]KG02994* obtained from Bloomington Stock Center. *KG02994* was crossed with *y,w;CyO, H{w[+mC]=PΔ2-3}HoP2.1/Bc,Egfr* to induce the mobilization of the KG02994 P-element. Mobilized P elements were identified among progeny by loss of yellow, white, or both markers. 126 candidates were characterized at the *dronc* locus by genomic PCR using primers flanking *dronc* (Figure 1A, open arrowhead primers 4 and 5; also 1C). The sequence for forward primer 4 is GTCAATGCCGTTCAGT TCCGTTGGCGATGGCT and reverse primer 5 is AAGATCGAGTTCA AGAGGACCAGCAAACTCAG. The PCR fragment from line 51 (*dronc*^{51}) genomic DNA was fully sequenced. The sequence at the junction is GTTTGTTAGGCTGACCCATGATGAAATAACATGTTAACA TAACAAATGTCCTTATTT. Genomic PCR fragments from two precise excision revertant lines, *dronc*^{rev78} and *dronc*^{rev79}, were also sequenced.

Complementation

Dronc rescue fragment transgenes, drf#4 and drf#8, were crossed into the *dronc*^{51} background and recombined to generate *dronc*$^{51:drf}$, which refers to drf#4, drf#8/+; *dronc*^{51}/*dronc*^{51}. While pharate adults occurred at frequencies up to ~50% of the expected Mendelian ratios, the number of eclosed adults were no more than 5% of the expected numbers. The drf transgene completely rescued the *hid* phenotype and enabled hatching in at least 10% of otherwise arrested embryos from *dronc*^{51}/KG02994 parents. We also tested a fragment that rescues CG6685 only (see Daish et al., 2004) and determined that this construct does not affect the latest lethal phase of *dronc*^{51} homozygotes.

Other Fly Strains

Df(3L) AC1,rn,p/TM3,Sb and *Hml-UAS:GAL4-GFP* were obtained from Bloomington Stock Center. *p[1.0Slit-LacZ]* (Ma et al., 2000) was generously provided by John Nambu. The method for producing wing clones were as in Vegh and Basler (2003). FRT-dronc^{51} refers to *Vg-GAL4:UAS-FLP / +; FRT79/ dronc*^{51} *FRT79* and FRT refers to *Vg-GAL4:UAS-FLP / +; FRT79/ FRT79*. The method for producing eye clones was adopted from Stowers and Schwarz (1999). In this case, FRT-dronc^{51} refers to *ey-GAL4:UAS-FLP/+; GMR-hid l(3)CL-L[1] FRT79/dronc*^{51} *FRT79* and FRT refers to *ey-GAL4:UAS-FLP/+; GMR-hid l(3)CL-L[1] FRT79/FRT79*.

RT-PCR

To access mRNA levels of *dronc* and CG6685 in *dronc*^{51} animals, total RNA was prepared from *dronc*^{51} and *yw* L3 larvae using High Pure RNA Isolation Kit (Roche). Superscript One-Step RT-PCR System w/ Platium Taq (Invitrogen) was used for RT-PCR reactions. *wgn* was used as an internal control for RT-PCR reactions.

Caspase Assays

Wild-type and mutant larval extracts were prepared by homogenizing about 30 L3 larvae in 5 ml of buffer A (20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) supplemented with 0.5% Triton-X 100 using a Teflon homogenizer. The soluble fraction was obtained by a 15 min spin at 15,000 $\times$ g at 4°C. 50 μg of protein extract was incubated with 10 μM Ac-DEVD-AFC or Ac-TQTD-AFC (Calbiochem) substrate in a final volume of 100 μl in a 96 microtiter plate. Fluorescence was monitored over time with excitation at 400 nm and emission at 505 nm in a SpectraFluor Plus plate reader. For hemocyte lysates, cells were prepared as described in the "Ex Vivo Hemocyte Analysis" section. Hemocytes were treated with CHX for 8 hr, then lysed in buffer A with 0.5% TX-100. The DEVDase assay was carried out as above, except that 10 μg protein was used. For the PARP cleavage assay, 10 μg hemocyte lysate was incubated with 1 μl in vitro translated ^{35}S-labeled PARP at 30°C for 1 hr. The reactions were stopped by adding SDS sample buffer. The proteins were resolved on 10% SDS-PAGE and transferred to PVDF membrane. The membrane was dried and exposed to phosphoimager.

Determination of Mutant Lethal Phase

0–1 hr embryo collections of *yw* or *yw;dronc*^{51}*/TM6,Tb,Hu* were collected and aged at 25°C for 36 hr before aliquots of ~100 larvae were transferred to fresh vials. The numbers of L2 and L3 larvae, pupae, and adults were followed daily from day 4. *Tb*$^{+}$ larvae were staged by their anterior spiracles morphology. Larvae were separated from food by resuspending the mixture in water, and 5 M NaCl was added gradually to float the larvae.

Third Instar Larva Dissections

Wandering L3 were dissected with fine dissecting forceps in a drop of phosphate-buffered solution (PBS) or 0.1 M phosphate buffer (72% Na_2HPO_4, 28% NaH_2PO_4). Dissected larval organs are fixed with 2% formaldehyde in PBS or 0.1 M phosphate buffer for 10 min and washed in PBS.

Immunohistochemistry

Embryos were collected and aged as in Rodriguez et al. (2002b). Fixed embryos in methanol or L3 larval eye discs in PBS are equilibrated to and washed in PBS with 0.1% Triton-X100 (PBT). PBT+NGS (PBT, 0.1% BSA, 5% normal goat serum) was used as a blocking solution for 1 hr before incubation with the primary antibody (1:600 guinea pig α-Kr [Kosman et al., 1998]; 1:500 α-Boss [Kramer et al., 1991]; 1:800, α-β-Gal, Promega) overnight at 4°C. The labeling was visualized with either Vectastain ABC peroxidase kit or fluorochrome labeled secondary antibodies (Vector Laboratories). Labeled samples were washed in PBS and mounted using Vectorshield (Vector Laboratories) on glass slides.

Microscopy and Imaging

All imaging was done on either an Axioscop microscope with an Axiocam MRC color digital camera (Zeiss) or a TCSSP Spectral Confocal Microscope (Leica). Scanning electron microscopy was done as in Chen et al. (1996), except that the samples were not fixed.

TUNEL Staining

Embryo TUNEL labeling is essentially as in White et al. (1994). In addition, the 3,3′-diaminobenzidine-peroxide signal was enhanced with NiCl and the embryos were precleared using 70% glycerol before imaging (Patel, 1994).

Hemocyte Density Counts

The assay for hemocyte density was modified from Asha et al. (2003). L3 larvae are submerged in halocarbon oil, and the body wall was carefully pierced with a tungsten needle to obtain a droplet of hemolymph. To calculate the number of cells per microliter of hemolymph, the hemolymph droplets from 3–15 larvae were combined into a single drop, and 1–4 μl of the hemolymph was withdrawn with a fine pipette and diluted with PBS for counting using a hemocytometer on a fluorescent inverting scope.

Ex Vivo Hemocyte Analyses

L3 instar larvae were washed thoroughly for 3 min in the following solutions: water, 70% ethanol, water, 50% bleach, water, 70% ethanol, and water again. Wild-type and mutant larvae were submerged and dissected in PBS. The liquid containing hemolymph was filtered with synthetic mesh (35XX Nitex) and washed 3 times with PBS, each time pelleting for 5 min at 1500 rpm. The aspirates were washed one final time with Schneider's media (GIBCO) supplemented with 10% FCS and 50 μg/ml gentamycin. The hemocytes were plated at densities of ~80,000–100,000 per well in 96-well plates with fresh media. For stress treatments, hemocyte aspirates were treated separately 1 hr after plating with the following agents at the concentrations noted: CHX (100 μM), actinomycin D (10 μM), etoposide (1 mM), ethanol (1%), DMSO (1%), and small molecule Smac mimetic (10 μM) (Li et al., 2004). Blebbing was used to quantify cell death and surviving cell counts were normalized to an untreated plate. Flow cytometry was conducted ~8 hr post treatment on a FACScan machine using Cell Quest software.

Acknowledgments

We are grateful to Misoo Kim and Emiko Sano for their dedicated assistance. We also thank Lai Wang for reagents, Konrad Basler for fly stocks, Helmut Kramer for the α-boss antibody, John Reinitz for the α-Kruppel antibodies, and Joachim Seeman for the use of his microscope. We also thank Charles Dearolf, Duojia Pan, and Robert Rawson for helpful discussions and Margaret Hickson for administrative support. This work was supported by the NIH (RO1GMO72124) grant.

Received: July 6, 2004
Revised: August 31, 2004
Accepted: September 20, 2004
Published: December 6, 2004

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